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501
CYSTEINE-DEPLETED MUTEINS
OF BIOLOGICALLY ACTIVE PROTEINS

SP
 CL $\frac{1}{2}$ Cross-reference to Related Application

P This application is a continuation-in-part
 5 of US Serial No. 486,162 filed (15 April 1983) ^{now abandoned} which is a
 continuation-in-part of US Serial No. 435,154 filed 19
 October 1982.

SU CL $\frac{1}{2}$ Description

P 10 Technical Field

L 10 This invention is in the general area of
 recombinant DNA technology. More specifically it
 relates to mutationally altered biologically active
 proteins that differ from their parent analogs by one
 or more substitutions/deletions of cysteine residues.

P 2015 Background Art

L Biologically active proteins that are micro-
 bially produced via recombinant DNA (rDNA) technology
 may contain cysteine residues that are nonessential to
 their activity but are free to form undesirable inter-

20 molecular or intramolecular links. One such protein

62 is microbially produced human beta interferon (IFN- β).

L In the course of the preparation of IFN- β by rDNA
 techniques, it has been observed that dimers and oli-

62 gomers of microbially produced IFN- β are formed in

25 E.coli extracts containing high concentrations of

62 IFN- β . This multimer formation renders purification

L and separation of IFN- β very laborious and time-con-
 suming and necessitates several additional steps in

30 the protein during purification and reoxidizing it to

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62 restore it to its original conformation, thereby
increasing the possibility of incorrect disulfide bond
formation. Furthermore, microbially produced IFN- β
has also been found to exhibit consistently low spec-
5 ific activity due perhaps to the formation of multi-
mers or of random intramolecular disulfide bridges.
It would be desirable, therefore, to be able to alter
62 microbially produced biologically active proteins such
as IFN- β in a manner that does not affect their activ-
10 ity adversely but reduces or eliminates their ability
to form intermolecular crosslinks or intramolecular
bonds that cause the protein to adopt an undesirable
tertiary structure (eg, a conformation that reduces
the activity of the protein).

15 The present invention is directed to produ-
cing by directed mutagenesis techniques mutationally
altered biologically active proteins (such proteins
are called "muteins", Glossary of Genetics and
Cytogenetics, 4th Ed, p 381, Springer-Verlag (1976))
20 that retain the activity of their parent analogs but
lack the ability to form intermolecular links or unde-
sirable intramolecular disulfide bonds. In this
regard Shepard, H.M., et al, Nature (1981) 294:563⁵⁶⁵
62 describe a mutein of IFN- β in which the cysteine at
25 position 141 of its amino acid sequence (there are
62 three cysteines in native human IFN- β at positions 17,
31, and 141, Gene (1980) 10:11¹⁵ and Nature (1980)
285:542⁵⁴⁷) is replaced by tyrosine. This mutein was
made by bacterial expression of a hybrid gene con-
62 30 structed from a partial IFN- β cDNA clone having a
62 G \rightarrow A transition at nucleotide 485 of the IFN- β
gene. The mutein lacked the biological activity of
62 native IFN- β leading the authors to conclude that the
replaced cysteine was essential to activity.

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Directed mutagenesis techniques are well known and have been reviewed by Lather, R.F. and Lecoq, J.P. in Genetic Engineering Academic Press (1983) pp 31¹/₅₀. Oligonucleotide-directed mutagenesis is specifically reviewed by Smith, M. and Gillam, S. in Genetic Engineering: Principles and Methods, Plenum Press (1981) 3:1¹/₃₂.

CL ¹/_c Disclosure of the Invention

^P One aspect of the invention is a synthetic
10 mutein of a biologically active protein which protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutein having at least one of said cysteine residues deleted or replaced by another amino
15 acid.

Another aspect of the invention relates to synthetic structural genes having DNA sequences that have been specifically designed ("designer genes") to encode the above described synthetic muteins. Sub-
20 aspects of this aspect are expression vectors that include such structural designer genes, host cells or organisms transformed with such vectors, and processes for making the synthetic mutein by culturing such transformants or their progeny and recovering the
25 mutein from the culture. In the case of muteins that have therapeutic utility, therapeutic compositions that contain therapeutically effective amounts of the muteins and therapeutic methods are other aspects of the invention.

30 Another aspect of the invention is a method of preventing a protein having one or more cysteine residues that is free to form an undesirable disulfide link from forming such a link comprising mutationally

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altering the protein by deleting the cysteine residue(s) or replacing them with other amino acids.

Still another aspect of the invention is a method for making the above described synthetic structural gene by oligonucleotide-directed mutagenesis comprising the following steps:

P (a) hybridizing single-stranded DNA comprising a strand of a structural gene that encodes the parent protein with a mutant oligonucleotide primer that is complementary to a region of the strand that includes the codon for the cysteine to be deleted or replaced or the antisense triplet paired with the codon, as the case may be, except for a mismatch with that codon or antisense triplet, as the case may be, that defines a deletion of the codon or a triplet that encodes said other amino acid;

P (b) extending the primer with DNA polymerase to form a mutational heteroduplex; and

P (c) replicating the mutational heteroduplex. The mutant oligonucleotide primers used in this process are another aspect of the invention.

DR CL 1/2

Brief Description of the Drawings

P Figure 1 is a diagram of the amino acid sequence of IFN- β .

25 Figure 2 is a schematic illustration showing the preparation of a mutant IFN- β gene by oligonucleotide-directed mutagenesis.

62 Figure 3 shows a diagram of plasmid p β ltrp including the IFN- β gene.

30 Figure 4 is a diagram of the cloning vector M13mp8 phage.

62 Figure 5 shows the restriction map of clone M13- β 1.

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62 Figure 6 shows the sequencing gel pattern of the mutant IFN- β_{ser17} gene showing a single base change in the coding region.

Figure 7 is a diagram of the expression
5 plasmid pTrp3.

Figure 8a shows the HinfI restriction pattern of clone pSY2501 and Figure 8b shows the resulting two 169bp and 28bp fragments thereof.

Figure 9 is a restriction map of clone
10 pSY2501.

62 Figure 10 shows the coding DNA sequence for the mutein IFN- β_{ser17} with the corresponding amino acid sequence therefor.

62 15 Figure 11 shows the single 18,000 dalton protein band corresponding to IFN- β_{ser17} in the extracts of clones pSY2501 and p β 1trp.

Figure 12 is a diagram of the plasmid pLW1 which contains the human interleukin-2 (IL-2) gene under the control of the E.coli trp promoter.

20 Figure 13 is a restriction map of phage clone M13-IL2.

Figure 14 is a restriction map of the plasmid pLW46.

25 Figures 15a and 15b show, respectively, the nucleotide sequence of the coding strand of the clone pLW46 and the corresponding amino acid sequence of the IL-2 mutein designated IL-2 $_{\text{ser125}}$.

Figure 16 is a diagram of the plasmid pLW32.

Figure 17 is a diagram of the plasmid pLW55.

DECL %³⁰ Modes for Carrying Out the Invention

P The present invention provides: muteins of biologically active proteins in which cysteine residues that are not essential to biological activity

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have been deliberately deleted or replaced with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation; mutant genes coding for such muteins;
5 and means for making such muteins.

Proteins that may be mutationally altered according to this invention may be identified from available information regarding the cysteine content of biologically active proteins and the roles played
10 by the cysteine residues with respect to activity and tertiary structure. For proteins for which such information is not available in the literature this information may be determined by systematically altering each of the cysteine residues of the protein by
15 the procedures described herein and testing the biological activity of the resulting muteins and their proclivity to form undesirable intermolecular or intramolecular disulfide bonds. Accordingly, while the invention is specifically described and exempli-
62 20 fied below as regards muteins of IFN- β and IL-2 it will be appreciated that the following teachings apply to any other biologically active protein that contains a functionally nonessential cysteine residue that makes the protein susceptible to undesirable disulfide
62 25 bond formation. Examples of proteins other than IFN- β and IL-2 that are candidates for mutational alteration according to the invention are lymphotoxin (tumor necrosis factor), colony stimulating factor-1, and
60 IFN- α 1. Candidate proteins will usually have an odd
30 number of cysteine residues.

62 In the case of IFN- β it has been reported in the literature and that both the glycosylated and unglycosylated IFNs show qualitatively similar specific activities and that, therefore, the glycosyl

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moieties are not involved in and do not contribute to the biological activity of IFN- β . However, bacterially produced IFN- β which is unglycosylated consistently exhibits quantitatively lower specific activity 5 than native IFN- β which is glycosylated. IFN- β is known to have three cysteine residues at positions 17, 31 and 141. Cysteine 141 has been demonstrated by Shepard, et al, supra, to be essential for biological activity. In IFN- α , which contains four cysteine 10 residues, there are two intramolecular S-S bonds: one between cys 29 and cys 138 and another between cys 1 and cys 98. Based on the homology between IFN- β and IFN- α s cys 141 of IFN- β could be involved in an intramolecular S-S bond with cys 31, leaving cys 17 15 free to form intermolecular crosslinks. By either deleting cys 17 or substituting it by a different amino acid, one can determine whether cys 17 is essential to biological activity, and its role in S-S bond formation. If cys 17 is not essential for the biolog- 20 ical activity of the protein, the resulting cys 17-deleted or cys 17-substituted protein might exhibit specific activity close to that of native 62 IFN- β and would possibly also facilitate isolation and purification of the protein.

25 By the use of the oligonucleotide-directed mutagenesis procedure with a synthetic oligonucleotide primer that is complementary to the region of the 62 IFN- β gene at the codon for cys 17 but which contains single or multiple base changes in that codon, a 30 designer gene may be produced that results in cys 17 being replaced with any other amino acid of choice. When deletion is desired the oligonucleotide primer lacks the codon for cys 17. Conversion of cys 17 to neutral amino acids such as glycine, valine, alanine,

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leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine and methionine is the preferred approach. Serine and threonine are the most preferred replacements because of their chemical analogy to cysteine. When the cysteine is deleted, the mature munein is one amino acid shorter than the native parent protein or the microbially produced IFN- β .

Human IL-2 is reported to have three cysteine residues located at positions 58, 105, and 125 of the protein. As in the case of IFN- β , IL-2 is in an aggregated oligomeric form when isolated from bacterial cells and has to be reduced with reducing agents in order to obtain a good yield from bacterial extracts. In addition, the purified reduced IL-2 protein is unstable and readily reoxidized upon storage to an oligomeric inactive form. The presence of three cysteines means that upon reoxidation, the protein may randomly form one of three possible intramolecular disulfide bridges, with only one of those being the correct bridge as found in the native molecule. Since the disulfide structure of the native IL-2 protein is not known, it is possible to use the present invention to create mutations at codons 58, 105 and 125 of the IL-2 gene and identify which cysteine residues are necessary for activity and therefore most likely to be involved in native disulfide bridge formation. In the same vein, the cysteine residue that is not necessary for activity can be modified so as to prevent the formation of incorrect intramolecular disulfide bridges and minimize the chance of intermolecular disulfide bridges by removal or replacement of the free cysteine residue.

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The size of the oligonucleotide primer is determined by the requirement for stable hybridization of the primer to the region of the gene in which the mutation is to be induced, and by the limitations of the currently available methods for synthesizing oligonucleotides. The factors to be considered in designing oligonucleotides for use in oligonucleotide directed mutagenesis (eg, overall size, size of portions flanking the mutation site) are described by Smith, M. and Gillam S., supra. In general the overall length of the oligonucleotide will be such as to optimize stable, unique hybridization at the mutation site with the 5' and 3' extensions from the mutation site being of sufficient size to avoid editing of the mutation by the exonuclease activity of the DNA polymerase. Oligonucleotides used for mutagenesis in accordance with the present invention usually contain from about 12 to about 24 bases, preferably from about 14 to about 20 bases and still more preferably from about 15 to about 18 bases. They will usually contain at least about three bases 3' of the altered or missing codon.

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The method for preparing the modified IFN- β gene broadly involves inducing a site-specific mutagenesis in the IFN- β gene at codon 17 (TGT) using a synthetic nucleotide primer which omits the codon or alters it so that it codes for another amino acid. When threonine replaces the cysteine and the primer is hybridized to the antisense strand of the IFN- β gene, the preferred nucleotide primer is CCAATTTTCAGCTCAG (underlining denotes the altered codon). When it is desirable to delete cysteine, the preferred primer is AGCAATTTTCAGCAGAAGCTCCTG, which omits the TGT codon for cysteine. When cysteine is replaced by serine, a

CCAATTTTCAGCTCAG
CCAATTTTCAGCTCAG

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43 17-nucleotide primer, GCAATTTTCAGAGTCAG, which includes an AGT codon for serine is the primer of choice. The T→A transition of the first base in the cys 17 codon results in changing cysteine to serine.

5 It must be recognized that when deletions are introduced, the proper reading frame for the DNA sequence must be maintained for expression of the desired protein.

518 10 The primer is hybridized to single-stranded phage such as M13, fd, or ØX174 into which a strand of 62 the IFN-β gene has been cloned. It will be appreciated that the phage may carry either the sense strand or antisense strand of the gene. When the phage carries the antisense strand the primer is identical to 15 the region of the sense strand that contains the codon to be mutated except for a mismatch with that codon that defines a deletion of the codon or a triplet that codes for another amino acid. When the phage carries the sense strand the primer is complementary to the 20 region of the sense strand that contains the codon to be mutated except for an appropriate mismatch in the triplet that is paired with the codon to be deleted. Conditions that may be used in the hybridization are described by Smith, M. and Gillam, S., supra. The 25 temperature will usually range between about 0°C and 70°C, more usually about 10°C to 50°C. After the hybridization, the primer is extended on the phage DNA by reaction with DNA polymerase I, T₄ DNA polymerase, reverse transcriptase or other suitable DNA polymer- 30 ase. The resulting dsDNA is converted to closed circular dsDNA by treatment with a DNA ligase such as T₄ DNA ligase. DNA molecules containing single-stranded regions may be destroyed by S1 endonuclease treatment.

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Oligonucleotide-directed mutagenesis may be similarly employed to make a mutant IL-2 gene that encodes a mutein having IL-2 activity but having cys 125 changed to serine 125. The preferred oligo-
5 nucleotide primer used in making this mutant IL-2 gene when the phage carries the sense strand of the gene is GATGATGCTTCTGAGAAAAGGTAATC. This oligonucleotide has
43 a C → G change at the middle base on the triplet that is paired with codon 125 of the IL-2 gene.

10 The resulting mutational heteroduplex is then used to transform a competent host organism or cell. Replication of the heteroduplex by the host provides progeny from both strands. Following repli-
15 cation the mutant gene may be isolated from progeny of the mutant strand, inserted into an appropriate expression vector, and the vector used to transform a suitable host organism or cell. Preferred vectors are plasmids pBR322, pCR1, and variants thereof, synthetic vectors and the like. Suitable host organisms are
20 E.coli, Pseudomonas, Bacillus subtilis, Bacillus thuringiensis, various strains of yeast, Bacillus thermophilus, animal cells such as mice, rat or Chinese hamster ovary (CHO) cells, plant cells, animal and plant hosts and the like. It must be recognized
25 that when a host of choice is transformed with the vector, appropriate promoter-operator sequences are also introduced in order for the mutein to be expressed. Hosts may be prokaryotic or eukaryotic (processes for inserting DNA into eukaryotic cells are des-
30 cribed in PCT applications nos US81/00239 and US81/00240 published (3 September 1981). E.coli and CHO cells are the preferred hosts. The muteins obtained in accordance with the present invention may be glycosylated or unglycosylated depending on the

glycosylation occurring in the native parent protein and the host organism used to produce the mutein. If desired, unglycosylated mutein obtained when E.coli or a Bacillus is the host organism, may be optionally glycosylated in vitro by chemical, enzymatic and other types of modifications known in the art.

62 In the preferred embodiment of the subject
L invention respecting IFN- β , the cysteine residue at
43 10 position 17 in the amino acid sequence of IFN- β , as
shown in Figure 1, is changed to serine by a T \rightarrow A
transition of the first base of codon 17 of the sense
strand of the DNA sequence which codes for the mature
62 IFN- β . The site-specific mutagenesis is induced using
a synthetic 17-nucleotide primer GCAATTTTCAGAGTCAG
15 which is identical to a seventeen nucleotide sequence
62 on the sense strand of IFN- β in the region of codon 17
except for a single base mismatch at the first base of
codon 17. The mismatch is at nucleotide 12 in the
primer. It must be recognized that the genetic code
20 is degenerate and that many of the amino acids may be
encoded by more than one codon. The base code for
serine, for example, is six-way degenerate such that
the codons, TCT, TCG, TCC, TCA, AGT, and ACG all code
for serine. The AGT codon was chosen for the prefer-
25 red embodiment for convenience. Similarly, threonine
is encoded by any one of codons ACT, ACA, ACC and
ACG. It is intended that when one codon is specified
for a particular amino acid, it includes all degen-
erate codons which encode that amino acid. The 17-mer
30 is hybridized to single-stranded M13 phage DNA which
62 carries the antisense strand of the IFN- β gene. The
oligonucleotide primer is then extended on the DNA
using DNA polymerase I Klenow fragment and the resul-
ting dsDNA is converted to closed circular DNA with T₄

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ligase. Replication of the resulting mutational heteroduplex yields clones from the DNA strand containing the mismatch. Mutant clones may be identified and screened by the appearance or disappearance of
5 specific restriction sites, antibiotic resistance or sensitivity, or by other methods known in the art.

43 When cysteine is substituted with serine, the T->A transition, shown in Figure 2, results in the creation of a new HinfI restriction site in the structural
10 gene. The mutant clone is identified by using the oligonucleotide primer as a probe in a hybridization screening of the mutated phage plaques. The primer will have a single mismatch when hybridized to the parent but will have a perfect match when hybridized
15 to the mutated phage DNA, as indicated in Figure 2. Hybridization conditions can then be devised where the oligonucleotide primer will preferentially hybridize to the mutated DNA but not to the parent DNA. The newly generated HinfI site also serves as a means of
20 confirming the single base mutation in the IFN- β gene.

The M13 phage DNA carrying the mutated gene is isolated and spliced into an appropriate expression vector, such as plasmid pTrp3, and E.coli strain MM294 is transformed with the vector. Suitable growth media
25 for culturing the transformants and their progeny are known to those skilled in the art. The expressed
62 mutein of IFN- β is isolated, purified and characterized.

The following examples are presented to help
30 in the better understanding of the subject invention and for purposes of illustration only. They are not to be construed as limiting the scope of the invention in any manner. Examples 1 $\frac{1}{N}$ 11 describe the preparation
62 of a mutein of IFN- β . Examples 12 $\frac{1}{N}$ 20 describe the
35 preparation of a mutein of IL-2.

CL v/c

Example 1

P 62 Cloning of the IFN- β Gene Into M13 Vector:

- The use of M13 phage vector as a source of single-stranded DNA template has been demonstrated by
- 5 G.F. Temple et al, Nature (1982) 296:537-540. Plasmid p β ltrp (Figure 3) containing the IFN- β gene under control of E.coli trp promoter, was digested with the restriction enzymes HindIII and XhoII. The M13mp8 (J. Messing, "Third Cleveland Symposium on
- 10 Macromolecules: Recombinant DNA," Ed. A Walton, Elsevier Press, 143-153 (1981)) replicative form (RF) DNA (Figure 4) was digested with restriction enzymes
- 62 HindIII and BamHI, and mixed with the p β ltrp DNA which had previously been digested with HindIII and XhoII.
- 15 The mixture was then ligated with T₄ DNA ligase and the ligated DNA transformed into competent cells of E.coli strain JM 103 and plated on Xgal indicator plates (J. Messing, et al, Nucleic Acids Res (1981) 9:309-321). Plaques containing recombinant phage
- 20 (white plaques) were picked, inoculated into a fresh culture of JM 103 and minipreps of RF molecules prepared from the infected cells (H.D. Birnboim and J. Doly, Nucleic Acid Res (1979) 7:1513-1523). The RF molecules were digested with various restriction
- 62 25 enzymes to identify the clones containing the IFN- β insert. The restriction map of one such clone
- 62 (M13-B1) is shown in Figure 5. Single-stranded (ss)
- L phage DNA was prepared from clone M13-B1 to serve as a template for site-specific mutagenesis using a syn-
- 30 thetic oligonucleotide.

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CL $\frac{1}{2}$

Example 2

Site-Specific Mutagenesis:

Forty picomoles of the synthetic oligonucleotide GCAATTTTCAGAGTCAG (primer) was treated with 5 T₄ kinase in the presence of 0.1 mM adenosine triphosphate (ATP), 50 mM hydroxymethylaminomethane hydrochloride (Tris-HCl) pH 8.0, 10 mM MgCl₂, 5 mM dithiothreitol (DTT) and 9 units of T₄ kinase, in 50 μ l at 37°C for 1 hr. The kinased primer (12 pmole) was hybridized to 5 μ g of ss M13-B1 DNA in 50 μ l of a mixture containing 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂ and 10 mM β -mercaptoethanol, by heating at 67°C for 5 min and at 42°C for 25 min. The annealed mixture was then chilled on ice and then added to 50 μ l of a reaction mixture containing 0.5 mM each of deoxynucleoside triphosphate (dNTP), 80 mM Tris-HCl, pH 7.4, 8 mM MgCl₂, 100 mM NaCl, 9 units of DNA polymerase I, Klenow fragment, 0.5 mM ATP and 2 units of T₄ DNA ligase, incubated at 37°C for 3 hr and at 25°C for 2 hr. The reaction was then terminated by phenol extraction and ethanol precipitation. The DNA was dissolved in 10 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 50% sucrose and 0.05% bromophenylblue and electrophoresed on 0.8% agarose gel in the presence of 2 μ g/ml of ethidium bromide. The DNA bands corresponding to the RF forms of M13-B1 were eluted from gel slices by the perchlorate method (R.W. Davis, et al, "Advanced Bacterial Genetics", Cold Spring Harbor Laboratory, N.Y., p. 178-179 (1980)). The eluted DNA was used to transform competent JM 103 cells, grown overnight and ssDNA isolated from the culture supernatant. This ssDNA was used as a template in a second cycle of

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primer extension, the gel purified RF forms of the DNA were transformed into competent JM 103 cells, plated onto agar plates and incubated overnight to obtain phage plaques.

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CL $\frac{1}{2}$ Example 3

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Site Specific Mutagenesis:

62 10 The experiment of Example 2 above is repeated except that the synthetic oligonucleotide primer used is GCAATTTTCAGACTCAG to change codon 17 of the IFN- β gene from one that codes for cysteine to one that codes for threonine.

CL $\frac{1}{2}$ Example 4

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Site Specific Deletion:

62 15 The experiment of Example 2 above is repeated except that the synthetic oligonucleotide primer used is AGCAATTTTCAGCAGAAGCTCCTG to delete codon 17 of the IFN- β gene.

CL $\frac{1}{2}$ Example 5

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Screening And Identification of Mutagenized Plaques:

62 20 62 Plates containing mutated M13- β 1 plaques (Example 1) as well as two plates containing unmutated M13- β 1 phage plaques, were chilled to 4°C and plaques from each plate transferred onto two nitrocellulose filter circles by layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2 N NaOH, 1.5 M NaCl and 0.2% Triton X-100 for 5 min, and neutralized by

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layering onto filter papers soaked with 0.5 M Tris-HCl, pH 7.5 and 1.5 M NaCl for another 5 min. The filters were washed in a similar fashion twice on filters soaked in 2 x SSC (standard saline citrate), dried and then baked in a vacuum oven at 80°C for 2 hr. The duplicate filters were prehybridized at 55°C for 4 hr with 10 ml per filter of DNA hybridization buffer (5 x SSC) pH 7.0, 4 x Denhardt's solution (polyvinylpyrrolidone, ficoll and bovine serum albumin, 1 x = 0.02% of each), 0.1% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate buffer pH 7.0 and 100 µg/ml of denatured salmon sperm DNA.

32p-labeled probe was prepared by kinasing the oligonucleotide primer with ³²P-labeled ATP. The filters were hybridized to 3.5 x 10⁵ cpm/ml of ³²P-labeled primer in 5 ml per filter of DNA hybridization buffer at 55°C for 24 hr. The filters were washed at 55°C for 30 min each in washing buffers containing 0.1% SDS and decreasing amounts of SSC.

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20 The filters were washed initially with buffer containing 2 x SSC and the control filters containing unmutated M13-β1 plaques were checked for the presence of any radioactivity using a Geiger counter. The concentration of SSC was lowered stepwise and the filters

62 25 washed until no detectable radioactivity remained on the control filters with the unmutated M13-β1 plaques.

33 33 The lowest concentration of SSC used was 0.1 x SSC. The filters were air dried and autoradiographed at

31 20 62 -70°C for 2 1/3 days. 480 plaques of mutated M13-β1 and 30 100 unmutated control plaques were screened with the

62 kinased oligonucleotide probe. None of the control plaques hybridized with the probe while 5 mutated M13-β1 plaques hybridized with the probe.

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One of the five mutated M13-β1 plaques (M13-SY2501) was picked and inoculated into a culture of JM 103. ssDNA was prepared from the supernatant and double-stranded (ds) DNA was prepared from the cell pellet. The ssDNA was used as a template for the dideoxy-sequencing of the clone using the M13 universal primer. The result of the sequence analysis is shown in Figure 6, confirming that the TGT cys codon has been converted to an AGT ser codon.

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CL 1/2 Example 6

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62 Expression of Mutated IFN-β in E.coli:

RF DNA from M13-SY2501 was digested with restriction enzymes HindIII and XhoII and the 520 bp insert fragment purified on a 1% agarose gel. The plasmid pTrp3 containing the E.coli trp promoter (Figure 7) was digested with the enzymes HindIII and BamHI, mixed with the purified M13-SY2501 DNA fragment, and ligated in the presence of T₄ DNA ligase. The ligated DNA was transformed into E.coli strain MM294. Ampicillin resistant transformants were screened for sensitivity to the drug tetracycline. Plasmid DNA from five ampicillin resistant, tetracycline sensitive clones were digested with HinfI to screen for the presence of the M13-SY2501 insert. Figure 8a shows the HinfI restriction pattern of one of the clones (pSY2501), comparing it with the HinfI pattern of the original IFN-β clone, pβ1trp. As expected, there is an additional HinfI site in pSY2501, cleaving the 197 bp IFN-β internal fragment to a 169 bp fragment and a 28 bp fragment (Figure 8b). A restriction map of the clone pSY2501 is shown in Figure 9. The complete DNA sequence of the mutant

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62 IFN- β gene is shown in Figure 10 together with the predicted amino acid sequence.

The plasmid designated as clone pSY2501 was deposited with the Agricultural Research Culture
5 Collection (NRRL), Fermentation Laboratory, Northern Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 60604 on 30
March³⁰ 1983 and was assigned accession numbers CMCC
10 No. 1533 and NRRL No. B-15356.

62 Cultures of pSY2501 and pBltrp, which include progeny thereof, were grown up to an optical density (OD₆₀₀) of 1.0. Cell free extracts were
15 prepared and the amount of IFN- β antiviral activity assayed on GM2767 cells in a microtiter assay.

62 Extracts of clone pSY2501 exhibited three to ten times higher activity than pBltrp (Table 1), indicating that
62 clone pSY2501 was either synthesizing more protein exhibiting IFN- β activity or that the protein made had
20 a higher specific activity.

Table 1

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	<u>EXTRACT</u>	<u>ANTIVIRAL ACTIVITY (U/ml)</u>
	pSY2501	6×10^5
	pBltrp	1×10^5
25	ptrp3 (control)	30

P In order to determine if clone pSY2501 was synthesizing several times more active protein, the extracts of both clones were electrophoresed on a SDS polyacrylamide gel together with a control extract and
30 the gel stained with coomassie blue to visualize the proteins. As shown in Figure 11, there was only one

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protein band corresponding to an apparent 18,000 dalton protein that was present in the extracts of clones pSY2501 and p β ltrp but not in the control extract of ptrp3. This protein, which has a molecular weight of about 20,000 daltons but shows a gel migration pattern of an 18,000 dalton protein was previously shown to be IFN- β by purification of this protein from extracts of p β ltrp. Since there is less of this protein in extracts of pSY2501 than in extracts of p β ltrp, the specific activity of the protein in extracts of clone pSY2501 was higher than that of clone p β ltrp.

CL $\frac{1}{2}$ Example 7

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15 The plasmid pSY2501 was transformed into a competent subvariant of E.coli strain MM294, designated MM294-1. A sample of the resulting transformant was deposited in the American Type Culture Collection 12301 Parklawn Drive, Rockville, Maryland 20852 USA on (18 November) 1983 under ATCC number 39,517.

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CL $\frac{1}{2}$ Example 8

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Production of IFN- β _{ser17}:

IFN- β _{ser17} was recovered from E.coli that had been transformed to produce IFN- β _{ser17}. The E.coli were grown in the following growth medium to an OD of 10^{-11} at 680 nm (dry wt 8.4 g/l).

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	Ingredient	Concentration
	NH ₄ Cl	20 mM
	K ₂ SO ₄	16.1 mM
	KH ₂ PO ₄	7.8 mM
5	Na ₂ HPO ₄	12.2 mM
	MgSO ₄ · 7H ₂ O	3 mM
	Na ₃ citrate · 2H ₂ O	1.5 mM
	MnSO ₄ · 4H ₂ O	30 µM
	ZnSO ₄ · 7H ₂ O	30 µM
10	CuSO ₄ · 5H ₂ O	3 µM
	L-tryptophan	70 mg/l
	FeSO ₄ · 7H ₂ O	72 µM
	thiamine · HCl	20 mg/l
	glucose	40 g/l

15 pH control with NH₄OH

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A 9.9 l (9.9 kg) harvest of the transformed E.coli was cooled to 20°C and concentrated by passing the harvest through a cross-flow filter at an average pressure drop of ~110 kpa and steady-state filtrate flow rate of 260 ml/min until the filtrate weight was 8.8 kg. The concentrate (approximately one liter) was drained into a vessel and cooled to 15°C. The cells in the concentrate were then disrupted by passing the concentrate through a Manton-Gaulin homogenizer at 5°C, ~69,000 kpa. The homogenizer was washed with one liter phosphate buffered saline, pH 7.4 (PBS), and the wash was added to the disruptate to give a final volume of two liters. This volume was continuously centrifuged at 12000 x g at a 50 ml/min flow rate. The solid was separated from the supernatant and resuspended in four liters PBS containing 2% by wt SDS. This suspension was stirred at room temperature for 15 min after which there was no visible suspended mater-

22

ial. The solution was then extracted with 2-butanol at a 1:1 2-butanol:solution volume ratio. The extraction was carried out in a liquid-liquid phase separator using a flow rate of 200 ml/min. The organic phase was then separated and evaporated to dryness to yield 21.3 g of protein. This was resuspended in distilled water at a 1:10 volume ratio.

62 The recovered product was assayed for human
10 IFN- β activity using an assay based on protection
82 against viral cytopathic effect (CPE). The assay was
L made in microtiter plates. Fifty μ l of minimum essential medium were charged into each well and 25 μ l of the sample was placed in the first well and 1:3 volume dilutions were made serially into the following wells.
15 Virus (vesicular stomatitis), cell (human fibroblast line GM-2767), and reference IFN- β controls were included on each plate. The reference IFN- β used was 100 units per ml. The plates were then irradiated with UV light for 10 min. After irradiation 100 μ l of
62 the cell suspension (1.2×10^5 cells/ml) was added to
L each well and the trays were incubated for 18^h/₂₄ hr.
82 A virus solution at one plaque-forming unit per cell
33 20 was added to each well except the cell control. The trays were then incubated until the virus control
25 showed 100% CPE. This normally occurred 18^h/₂₄ hr after adding the virus solution. Assay results were interpreted in relation to the location of the 50% CPE
62 well of the reference IFN- β control. From this point
the titer of interferon for all samples on the plate
30 was determined. The specific activity of the recovered product was determined to be 5×10^7 U/mg.
33

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CL %

Example 9

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Acid Precipitation And Chromatographic Purification;

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The process of Example 8 was repeated except that after extraction and separation of the aqueous and organic phases and mixing of the organic phase with PBS at a volume ratio of 3:1 the pH of the mixture was lowered to about 5 by addition of glacial acetic acid. The resulting precipitate was separated by centrifugation at 10000-17000 x g for 15 min and the pellet was redissolved in 10% w/v SDS, 10 mM DTT, 50 mM sodium acetate buffer, pH 5.5, and heated to 80°C for 5 min.

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The solution was then applied to a Brownlee RP-300, 10 μ M, "Aquapore" column using a Beckman gradient system. Buffer A was 0.1% trifluoroacetic acid (TFA) in H₂O; buffer B was 0.1% TFA in acetonitrile. Detection was by ultraviolet absorbance at 280 nm. The solvent program was linear gradient of 0% buffer B to 100% buffer B in three hr. Fractions containing highest interferon activities were pooled and the specific activity of the pooled interferon preparation was determined to be 9.0×10^7 to 3.8×10^8 international units per mg protein, as compared to about 2×10^8 U/mg for native IFN- β .

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CL %

Example 10

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Biochemical Characterization of IFN- β Ser₁₇*

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Amino acid compositions were determined after 24-72 hr timed hydrolysis of 40 μ g samples of IFN in 200 μ l of 5.7 N HCl, 0.1% phenol, at 108°C. Proline and cysteine were determined in the same fashion after performic acid oxidation; in this case,

24

82 phenol was omitted from the hydrolysis. Tryptophan was analyzed after 24 hr hydrolysis of 400 µl samples in 5.7 N HCl, 10% mercaptoacetic acid (no phenol). Analysis was performed on a Beckman 121MB amino acid 5 analyzer using a single column of AA10 resin.

62 The amino acid composition calculated from representative 24-, 48-, 72-hr acid hydrolyses of purified IFN-β Ser₁₇ agrees well with that predicted by the DNA sequence of the cloned IFN gene, minus the 10 missing N-terminal methionine.

33 20 The amino acid sequence of the first 58 residues from the amino acid terminus of purified IFN was determined on a 0.7 mg sample in a Beckman 890C sequanator with 0.1 M Quadrol buffer. PTH amino acids 15 were determined by reverse-phase HPLC on an Altex 20 ultrasphere ODS column (4.6 x 250 mm) at 45°C eluted at 1.3 min at 40% buffer B, and 8.4 min from 40% 70% buffer B, where buffer A was 0.0115 M sodium acetate, 5% tetrahydrofuran (THF), pH 5.11 and buffer B was 10% 20 THF in acetonitrile.

62 The N-terminal amino acid sequence of IFN-β Ser₁₇ determined matches the expected sequence predicted from the DNA sequence, except for the absence of N-terminal methionine.

25

CL 1/2 Example 11

P 62 Alternative IFN-β_{ser} Production and Purification Process;

P E.coli transformed with pSY2501 were grown in the following medium:

25

T0260X

	<u>Ingredient</u>	<u>Approximate Initial Concentration</u>
	Na ₃ Citrate · 2H ₂ O	3 mM
	KH ₂ PO ₄	30 mM
	(NH ₄) ₂ SO ₄	74 mM
5	MgSO ₄ · 7H ₂ O	3 mM
	MnSO ₄ · H ₂ O	46 μM
	ZnSO ₄ · 7H ₂ O	46 μM
	CuSO ₄ · 5H ₂ O	1-2 μM
	L-tryptophan	350 μM
10	FeSO ₄ · 7H ₂ O	74 μM
	thiamine · HCl	0.002%
	glucose	0.5%

P

Dow Corning Antifoam polypropylene glycol, 25% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

Temperature was maintained at 37 ±1°C, pH at 6.5 ±0.1 with NaOH, and dissolved oxygen at 30% of air saturation. Optical density and residual glucose measurements were taken at 14 hr and at approximately one hr intervals thereafter. Harvest was made when glucose consumption reached 40 ±6 g/l (OD at 680 nm = 10^{-1.11}).

The harvested material was concentrated approximately 3-fold by circulating it through a microporous cross-flow filter under pressure. The concentrated cells were diafiltered against deionized water until the harvest material was concentrated 4¹/₅ fold. The cells were then disrupted by passing them through a Manton-Gaulion homogenizer at 4.1 - 5.5 x 10⁴ kpa. After the initial pass SDS-sodium phosphate buffer was added to a final concentration of 2% SDS, 0.08 M sodium phosphate and homogenization was

26

35 20 continued for one hr. Solid DTT was then added to a final concentration of 50 mM and the homogenizate was heated to $90 \pm 5^\circ\text{C}$ for 10 min. The resulting cell suspension was extracted with 2-butanol at a 1:1

5 2-butanol:suspension volume ratio in a static mixer. The mixture was then centrifuged and the 2-butanol rich phase was collected.

10 The 2-butanol rich phase was mixed with 2.5 volumes of 0.1% SDS in PBS. Solid DTT was added to a final concentration of 2 mM. The pH of the mixture was adjusted to 6.2 ± 0.1 with glacial acetic acid and this mixture was centrifuged. The resulting paste was collected and resuspended in PBS +10% SDS with pH adjustment to 8.5 ± 0.1 using 1 N NaOH. Solid DTT was added to a final concentration of 100 mM and the suspension was heated to $90 \pm 5^\circ\text{C}$ for 10 min. The suspension was then cooled to 25°C , the pH was adjusted to 5.5 ± 0.1 with glacial acetic acid, and the solution was filtered.

20 The solution was then applied to a Sephacryl S-200 pre column and the fractions containing highest interferon activities were pooled and concentrated by ultrafiltration with a 10 Kdal molecular weight cut-off. The concentrate was oxidized by adding equimolar amounts of protein and iodosobenzoic acid into a reaction vessel containing 2 mM sodium pyrophosphate, 0.1% SDS and 1 mM EDTA. The pH was controlled during oxidation at 9.0 ± 0.1 with 0.5 N NaOH and adjusted to 5.5 ± 0.2 when oxidation was complete. After oxidation the concentrate was again passed through the ultrafiltration unit with a 10 Kdal molecular weight cutoff.

35 L The concentrate was applied to a main Sephacryl S-200 column and the fractions were analyzed

27

by SDS-PAGE to determine those containing no high molecular weight contaminants. Those fractions were pooled and passed through the ultrafiltration unit. The filtered concentrate was then fractionated on a 5 Sephadex G-75 column. SDS-PAGE analysis of the fractions was made to determine those containing no low or high molecular weight contaminants. Those fractions were pooled for desalting.

10 A Sephadex G-25 column equilibrated with 1 mM NaOH was loaded with the pooled fractions from the Sephadex G-75 column using distilled water adjusted to pH 10.8-11 with 50% NaOH. The purified product was collected as the void volume peak. This desalted, 62 purified IFN- β mutein may be formulated in known manners for therapeutic administration. 15

P 62 Biological Testing of IFN- β_{ser17} :

L Antigenic Comparison:

62 IFN- β_{ser17} was compared antigenically to 62 IFN- β produced from diploid fibroblasts using virus 20 neutralizing tests. A polyvalent antiserum to the 62 diploid fibroblast IFN- β was prepared in rabbits. This antiserum blocked the antiviral activity of both the diploid fibroblast IFN- β and the IFN- β_{ser17} in the virus neutralization tests, indicating the two proteins are indistinguishable antigenically. 25

P Antiviral Activity:

L 62 The purified IFN- β_{ser17} was compared in its 62 antiviral activity to naturally produced IFN- β . Inhibition of vesicular stomatitis virus replication in 30 diploid foreskin fibroblast (HS27F) was indistinguishable from that of the natural molecule. Similarly,

inhibition of herpes simplex virus type 1 in HS27F fibroblasts by the natural and mutant proteins were comparable.

P 62 Antiproliferative Activity:

5 L The antiproliferation activity of IFN- β_{ser17} for continuous cell lines was compared with that of naturally produced IFN- β . T24 cells derived from a transitional cell carcinoma were treated with 200 units/ml of the proteins. Cell growth was inhibited 10 significantly ($p < 0.02$) by both proteins.

P L 62 Natural Killer (NK) Cell Stimulation:

62 20 The ability of IFN- β_{ser17} to stimulate NK cell (spontaneous cell mediated cytotoxicity) activity was tested. Ficoll-hypaque separated peripheral human 15 mononuclear cells (PMC) or NK-enriched lymphocyte preparations (depleted of monocytes by plastic adherence and of OKT3-positive T cells by treatment with OKT3 antibody plus complement) were incubated overnight in growth medium containing various concentrations of IFN- β_{ser17} . ^{51}Cr -labeled target cells were incubated with the effector cells (effector cell:target cell ratio = 50:1) for $2\frac{1}{4}$ hours. NK cell cytotoxicity was determined by measuring the amount of label released into the medium. The results of these tests 25 are reported in Table I below.

T0300X

TABLE I

NK Cell Cytotoxicity by Interferon (specific 51 Cr release \pm SEM)

Target Cell	Effector Cells	IFN (units/ml)				
		0	10	30	100	300
T24	PMC	7.23 \pm 5.1	23.1 \pm 4.4	24.4 \pm 1.1	34.1 \pm 2.5	50.0 \pm 2.0
Chang	PMC	4.7 \pm 0.5	7.2 \pm 0.8	9.5 \pm 1.7	15.9 \pm 1.3	21.9 \pm 1.4
Chang	NK Enr	19.2 \pm 4.6	39.4 \pm 4.1	ND	54.2 \pm 6.1	ND
K562	NK Enr	41.0 \pm 4.6	48.4 \pm 3.6	ND	62.2 \pm 3.5	ND
						40.4 \pm 4.4
						26.9 \pm 1.8
						41.7 \pm 5.5
						63.2 \pm 3.5

30

P
62 As shown the target cells were killed more
12 effectively by the IFN- β _{ser17}-treated cells than by
the ~~untreated~~ ^{untreated} cells.

P
5 L Clinical Trials:

62 Phase I clinical trials to verify the safety
of IFN- β _{ser17} in humans have been initiated. These
trials involve administering the protein to patients
intramuscularly and intravenously at doses ranging
33 42 between 1×10^5 units ($1 \mu\text{g}$ of protein) to
10 400 $\times 10^6$ units. In initial phase I clinical trials
no unexpected adverse effects have occurred.

62 As indicated above, the IFN- β _{ser17} prepara-
tion exhibits specific activity levels very close to
or better than that of native IFN- β . IFN- β _{ser17} has
15 no free sulfhydryl groups but indicates one ~~S-S~~ ^{disulfide} bond
between the only remaining cysteines at positions 31
and 141. The protein does not readily form oligomers
and appears to be substantially in the monomeric form.

62 The IFN- β _{ser17} obtained in accordance with this inven-
20 tion may be formulated either as a single product or
mixtures of the various forms, into pharmaceutically
acceptable preparations in inert, nontoxic, nonaller-
genic, physiologically compatible carrier media for
clinical and therapeutic uses in cancer therapy or in
25 conditions where interferon therapy is indicated and
for viral infections such as herpes simplex virus I
and II, hepatitis B virus, common cold viruses, and
rhinovirus. Such media include but are not limited to
3 distilled water, physiological saline, Ringer's solu-
30 tion, Hank's solution and the like. Other nontoxic
stabilizing and solubilizing additives such as dex-
trose, HSA (human serum albumin) and the like may be
c optionally
c optimally included. The therapeutic formulations may

be administered orally or parenterally such as intra-
venous, intramuscular, intraperitoneal and subcuta-
neous administrations. Preparations of the modified
IFN- β of the present invention may also be used for
5 topical applications in appropriate media normally
utilized for such purposes. The IFN- β mutein may be
administered either locally or systemically by itself
or in combination or conjunction with other therapeu-
tic agents such as ^{cyclosporin} cyclosporin for prophylactic or
10 therapeutic purposes. ^{for example, U.S. Patent No. 4,353,032} The dose of mutein administered
to human patients will depend on whether it is
administered continuously (including intermittent) or
as a bolus. The amounts administered continuously
will typically be lower than the amounts administered
15 as a bolus. The amount will usually be in the range
of about 1×10^5 to 4×10^8 units, more usually about
 1×10^6 to 1×10^7 units.

The principal advantages of the above des-
cribed mutein of IFN- β lie in the elimination of a
20 free sulfhydryl group at position 17 in IFN- β , thereby
forcing the protein to form correct disulfide links
between cys 31 and cys 141 and to assume the confor-
mation ostensibly required for full biological activ-
ity. The increased specific activity of the
25 IFN- β_{ser17} enables the use of smaller dosages in
therapeutic uses. By deleting the cysteine at posi-
tion 17 and eliminating the free ^mSH group, the
IFN- β_{ser17} protein does not form dimers and oligomers
so readily as the microbially produced IFN- β . This
30 facilitates purification of the protein and enhances
its stability.

32

CL ^{1/2}

Example 12

P The nucleotide sequence for a cDNA clone coding for human IL-2, procedures for preparing IL-2 cDNA libraries, and screening same for IL-2 are described by Taniguchi, T., et al, Nature (1983) Vol 24,
5 p 305 et seq.

cDNA libraries enriched in potential IL-2 cDNA clones were made from an IL-2 enriched mRNA fractions obtained from induced peripheral blood
10 lymphocytes (PBL) and Jurkat cells by conventional procedures. The enrichment of the mRNA for IL-2 message was made by fractionating the mRNA and identifying the fraction having IL-2 mRNA activity by injecting the fractions in Xenopus laevis oocytes and
15 assaying the oocyte lysates for IL-2 activity on HT-2 cells (J. Watson, J Exp Med (1979) 150:1570-1519 and S. Gillis et al, J Immun (1978) 120:2027-2032.)

CL ^{1/2}

Example 13

Screening and Identification of IL-2 cDNA Clones:

P
L 20 The IL-2 cDNA libraries were screened using the colony hybridization procedure. Each microtiter plate was replicated onto duplicate nitrocellulose filter papers (S & S type BA-85) and colonies were
20 allowed to grow at 37°C for 14-16 hr on L agar containing 50 µg/ml ampicillin. The colonies were lysed
25 and DNA fixed to the filter by sequential treatment for 5 min with 500 mM NaOH, 1.5 M NaCl, washed twice
3- for 5 min each time with 5 x standard saline citrate
20 (SSC). Filters were air dried and baked at 80°C for 2
L 30 hr. The duplicate filters were pre-hybridized at 42°C for 6-8 hr with 10 ml per filter of DNA hybridization

33

33 3 buffer (50% formamide, 5 x SSC, pH 7.0, 5 x Denhardt's solution (polyvinylpyrrolidone, plus ficoll and bovine serum albumin; 1 x = 0.2% of each), 50 mM sodium phosphate buffer at pH 7.0, 0.2% SDS, 20 µg/ml Poly U, and 50 µg/ml denatured salmon sperm DNA.

A ³²P-labeled 20-mer oligonucleotide probe was prepared based on the IL-2 gene sequence reported by Taniguchi, T., et al, supra. The nucleotide sequence of the probe was GTGGCCTTCTTGGGCATGTA.

10 20 The samples were hybridized at 42°C for 24-36 hr with 5 ml/filter of DNA hybridization buffer containing the ³²P-oligonucleotide probe. The filters were washed two times for 30 min each time at 50°C with 2 x SSC, 0.1% SDS, then washed twice with 1 x SSC and 0.1% SDS at 50°C for 90 min, air dried, and autoradiographed at -70°C for 2 to 3 days. Positive clones were identified and rescreened with the probe. Full length clones were identified and confirmed by restriction enzyme mapping and comparison with the 20 sequence of the IL-2 cDNA clone reported by Taniguchi, T., et al, supra.

CL %

Example 14

Cloning of IL-2 Gene into M13 Vector:

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L
25 The IL-2 gene was cloned into M13mp9 as described in Example 1 using the plasmid pLW1 (Figure 12) containing the IL-2 gene under the control of the E.coli trp promoter. A sample of pLW1 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA, on 30 4 August 1983 and has been assigned ATCC number 39,405. The restriction map of one clone (designated M13-IL2) containing the IL-2 insert is shown in

Figure 13. Single-stranded phage DNA was prepared from clone M13-IL2 to serve as a template for oligonucleotide-directed mutagenesis.

CL $\frac{1}{2}$ c Example 15

P
L

5 Oligonucleotide-directed Mutagenesis:

As indicated previously, IL-2 contains cysteine residues at amino acid positions 58, 105 and 125. Based on the nucleotide sequences of the portions of the IL-2 gene that contain the codons for these three cysteine residues three oligonucleotide primers were designed and synthesized for mutating the codons for these residues to codons for serine. These oligonucleotides have the following sequences.

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L

- CTTCTAGAGACTGCAGATGTTTC (DM27) to change cys 58,
15 CATCAGCATACTCAGACATGAATG (DM28) to change cys 105 and
GATGATGCTCTGAGAAAAGGTAATC (DM29) to change cys 125.

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20 units of T₄ kinase in 50 μ l at 37°C for 1 hr. Each of the kinased primers (10 pmoles) was hybridized to 2.6 μ g of ss M13-IL2 DNA in 15 μ l of a mixture containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.9, 20 mM MgCl₂ and 20 mM β -mercaptoethanol, by heating at 67°C for 5 min and 42°C for 25 min. The annealed mixtures were chilled on ice and then adjusted to a final, volume of 25 μ l of a reaction mixture containing 0.5 mM of each dNTP, 17 mM Tris-HCl, pH 7.9, 17 mM MgCl₂, 83 mM NaCl, 17 mM β -mercaptoethanol, 5 units of DNA polymerase I Klenow fragment, 0.5 mM ATP and 2

(82)

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20 units of T₄ DNA ligase, incubated at 37°C for 5 hr.
The reactions were terminated by heating to 80°C and
the reaction mixtures used to transform competent
JM103 cells, plated onto agar plates and incubated
5 overnight to obtain phage plaques.

CL % Example 14 16

P

Screening and Identification of Mutagenized Phage
Plaques:

P Plates containing mutagenized M13-IL2
10 plaques as well as 2 plates containing unmutagenized
M13-IL2 phage plaques, were chilled to 4°C and phage
plaques from each plate were transferred onto two
nitrocellulose filter circles by layering a dry filter
on the agar plate for 5 min for the first filter and
15 15 min for the second filter. The filters were then
placed on thick filter papers soaked in 0.2 N NaOH,
1.5 M NaCl and 0.2% Triton for 5 min, and neutralized
by layering onto filter papers soaked with 0.5 M
Tris-HCl, pH 7.5, and 1.5 M NaCl for another 5 min.
20 The filters were washed in a similar fashion twice on
filters soaked in 2 x SSC, dried and then baked in a
vacuum oven at 80°C for 2 hr. The duplicate filters
were pre-hybridized at 42°C for 4 hr with 10 ml per
3 filter of DNA hybridization buffer (5 x SSC, pH 7.0,
25 4 x Denhardt's solution (polyvinylpyrrolidone, ficoll
and bovin serum albumin, 1x = 0.02% of each), 0.1%
SDS, 50 mM sodium phosphate buffer, pH 7.0 and 100
µg/ml of denatured salmon sperm DNA. ³²P-labelled
probes were prepared by kinasing the oligonucleotide
30 primers with labelled ATP. The filters were hybri-
dized to 0.1 x 10⁵ cpm/ml of ³²P-labelled primers in
5 ml per filter of DNA hybridization buffer at 42°C

36

20 for 8 hr. The filters were washed twice at 50°C for
30 min each in washing buffers containing 0.1% SDS and
33 20 2 x SSC, and twice at 50°C for 30 min each with 0.1%
33 SDS and 0.2 x SSC. The filters were air dried and
31 20 5 autoradiographed at -70°C for 2¹/₃ days.

Since the oligonucleotide primers DM28 and
DM29 were designed to create a new DdeI restriction
site in the mutagenized clones (Figure 14), RF-DNA
from a number of the clones which hybridized with each
10 of these kinased primers were digested with the res-
triction enzyme DdeI. One of the mutagenized M13-IL2
placques which hybridized with the primer DM28 and has
a new DdeI restriction site (M13-LW44) was picked and
inoculated into a culture of JM103, ssDNA was prepared
15 from the culture supernatant and dsRF-DNA was prepared
from the cell pellet. Similarly, a plaque which
hybridized with primer DM29 was picked (M13-LW46) and
ssDNA and RF-DNA prepared from it. The oligonucleo-
tide primer DM27 was designed to create a new PstI
20 restriction site instead of a DdeI site. Therefore,
the plaques that hybridized to this primer were
screened for the presence of a new PstI site. One
such phage plaque was identified (M13-LW42) and ssDNA
and RF-DNA prepared from it. The DNA from all three
25 of these clones were sequenced to confirm that the
target TGT codons for cysteine had been converted to a
TCT codon for serine.

37

CL %

Example 17

P

Recloning of the Mutagenized IL-2 Gene for Expression in E.coli:

P RF-DNA from M13-LW42, M13-LW44 and M13-LW46
5 were each digested with restriction enzymes HindIII
and BanII and the insert fragments purified from a 1%
agarose gel. Similarly, the plasmid pTrp3 (Figure 7)
was digested with HindIII and BanII, the large plasmid
fragment containing the trp promoter was purified on
10 an agarose gel and then ligated with each of the
insert fragments isolated from M13-LW42, M13-LW44 and
M13-LW46. The ligated plasmids were transformed into
competent E.coli K12 strain MM294. The plasmid DNAs
from these transformants were analyzed by restriction
15 enzyme mapping to confirm the presence of the plasmids
pLW42, pLW44 and pLW46. Figure 14 is a restriction
map of pLW46. When each of these individual clones
were grown in the absence of tryptophane to induce the
trp promoter and cell free extracts analyzed on SDS-
20 polyacrylamide gels, all three clones, pLW42, pLW44
and pLW46, were shown to synthesize a 14.5 kd protein
similar to that found in the positive control, pLW21,
which has been demonstrated to synthesize a 14.4 kd
IL-2 protein. When these same extracts were subjected
25 to assay for IL-2 activity on mouse HT-2 cells, only
clones pLW21 (positive control) and pLW46 had signi-
ficant amounts of IL-2 activity (Table II below),
indicating that cys 58 and cys 105 are necessary for
biological activity and changing them to serines
30 (pLW42 and pLW44 respectively) resulted in the loss of
biological activity. Cys 125 on the other hand must
not be necessary for biological activity because
changing it to ser 125 (pLW46) did not affect the
biological activity.

38

T0390K

Table II

	Clones	IL-2 Activity (μ/ml)
	pIL2-7 (negative control)	1
	pLW21 (positive control)	113,000
5	pLW42	660
	pLW44	1,990
	pLW46	123,000

P Figure 15a shows the nucleotide sequence of the coding strand of clone pLW46. As compared to the coding strand of the native human IL-2 gene clone pLW46 has a single base change of G → C at nucleotide 374. Figure 15b shows the corresponding amino acid sequence of the IL-2 mutein encoded by pLW46. This mutein is designated ^{des-alanyl(ala)} IL-2_{ser125}. As compared to native IL-2 the mutein has a serine instead of a cysteine at position 125, has an initial N-terminal methionine (which is processed off), and lacks the initial N-terminal alanine of the native molecule.

A sample of E.coli K12 strain MM294 transformed with pLW46 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA on 26 September 1983 and has been assigned ATCC Number 39,452.

Examples 18 and 19 describe the preparation of an alternative and preferred vector for expressing

IL-2_{ser125}

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JFM 12/19/83
SDC 12/19/83

JFM 12/19/83
SDC 12/19/83

CL 1/2

Example 18

Construction of Ala-IL-2 Expression Vector pLW32:

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L
(40)

A codon (GCG) for alanine was inserted immediately after the initiation codon of the IL-2 gene of pLW1 by oligonucleotide-directed mutagenesis as follows. The oligonucleotide primer, 5'-GAAGTAGGCGCCATAAG-3', was kinased, hybridized to ssM13-IL2 DNA, and extended using the general procedure of Example 15 to form a mutational heteroduplex. In addition to the insertion of the GCG codon, the mutagenesis generated a new NarI restriction site in the gene. The heteroduplex was converted to closed circular heteroduplex and the circular heteroduplexes were used to transform competent JM103 cells and plated onto agar plates and incubated as in Example 15. The plates were screened to identify mutagenized M13-IL2 by the procedure of Example 16. One mutagenized phage, identified as M13-LW32, was selected for use in additional cloning and RF-DNA was prepared from it. Fig. 16 is a diagram of plasmid pLW32.

CL 1/2

Example 19

Construction of Ala-IL-2_{ser125} Expressing Clone pLW55:

P
L

RF-DNA from M13-LW46 (Examples 16 and 17) was digested with XbaI and PstI and the 530 bp fragment containing the carboxy terminal coding region of the IL-2_{ser125} gene was purified from an agarose gel. Similarly, pLW32 was digested with XbaI and PstI and the large fragment consisting of the plasmid vector and the ala-IL-2 N-terminal coding sequence was purified. The two purified DNA fragments were pooled and ligated using T₄ DNA ligase. The ligated DNA was

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transformed into competent E.coli K-12 strain MM294. Tetracycline resistant transformants were analyzed by restriction enzyme mapping for the presence of a plasmid containing an ala-IL-2_{ser125} gene, identified as 5 pLW55, which has a new DdeI site not found in pLW32. Fig 17 is a diagram of pLW55. Cell free extracts of bacterial culture containing pLW55 were found to contain over 10⁵ units of IL-2 activity per ml by the HT-2 cell assay, J. Watson, supra, and S. Gillis, 10 supra. Ala-IL-2_{ser125} protein is identical to the IL-2_{ser125} molecule shown in Fig 15(b) except that the former includes the initial N-terminal alanine of the native molecule.

A sample of E.coli K-12 strain MM294 15 transformed with pLW55 was deposited in the American Type Culture Collection on 18 November 1983 and has been assigned ATCC number 39,516.

CL $\frac{1}{2}$ Example 20

P
L

20

Ala-IL-2_{ser125} Production and Purification:

E.coli transformed with pLW55 were grown in a fermenter containing the following medium:

T0410X

c
c
c

25

(NH ₄) ₂ SO ₄	150 mM
KH ₂ PO ₄	21.6 mM
Na ₃ Citrate	1.5 mM
ZnSO ₄ · 7H ₂ O	30 μ M
MnSO ₄ · H ₂ O	30 μ M
CuSO ₄ · 5H ₂ O	1 μ M

pH adjusted to 6.50 with 2.5 N NaOH autoclaved

41

Sterile Additions (post autoclave)

	MgSO ₄ · 7H ₂ O	3 mM
	FeSO ₄	100 μM
	L-tryptophan	14 mg/l
5	Thiamine-HCl	20 mg/l
	Glucose	5 g/l
	Tetracycline	5 mg/l
	Ethanol	2%
	Casamino acids	2%

10 P Dow Corning Antifoam polypropylene glycol, 20% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

35 20 The pH of the fermenter was maintained at 6.8 with 5 N KOH. Residual glucose was maintained between 5-10 g/l, dissolved oxygen at 40%, and temperature at 37 ± 1°C. The casamino acids (20% stock solution) to a concentration of 2% were added when the OD₆₈₀ was about 10. Harvest was made three hr after the OD reached about 20.

20 The harvested material was concentrated and homogenized as in Example 11. Following DTT-heat treatment, the material was centrifuged and the resulting paste was extracted with urea to a final concentration of 4M. The suspension was centrifuged 25 and SDS was added to the solid phase to a concentration of 5%.

C PAGE 521 30 The solution was applied to a Sephacryl S-200 column and fractions containing IL-2 (by SDS-PAGE) were pooled. The pooled fractions were applied to a Whatman M-40 column packed with 18 micron Vydac C₄ 300 Å pore size bonded phase silica gel equilibrated in 0.1% TFA. The IL-2 mutein was eluted with a

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gradient of 40% to 60% 2-propanol, containing 0.1% TFA, in 160 min. Fractions containing highest IL-2 activities were pooled and found to have specific activities comparable to native IL-2.

- 5 Muteins of IL-2 in which the cysteine at position 125 has been deleted or replaced with another amino acid, such as the mutein IL-2_{ser125} retain IL-2 activity. They may, therefore, be formulated and used in the same manner as native IL-2. Accordingly, such
- 10 IL-2 muteins are useful for the diagnosis and treatment (local or systemic) of bacterial, viral, parasitic, protozoan and fungal infections; for augmenting cell-mediated cytotoxicity; for stimulating lymphokine activated killer cell activity; for mediating recovery
- 15 of immune function of lymphocytes; for augmenting alloantigen responsiveness; for facilitating recovery of immune function in acquired immune deficient states; for reconstitution of normal immunofunction in aged humans and animals; in the development of diag-
- 20 nostic assays such as those employing enzyme amplification, radiolabelling, radioimaging, and other methods known in the art for monitoring IL-2 levels in the diseased state; for the promotion of T cell growth in vitro for therapeutic and diagnostic purposes for
- 25 blocking receptor sites for lymphokines; and in various other therapeutic, diagnostic and research applications. The various therapeutic and diagnostic applications of human IL-2 have been investigated and reported in S.A. Rosenberg, E.A. Grimm, et al, A.
- 30 Mazumder, et al, and E.A. Grimm and S.A. Rosenberg. IL-2 muteins may be used by themselves or in combination with other immunologically relevant B or T cells or other therapeutic agents. Examples of relevant cells are B or T cells, natural killer cells, and the

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like and exemplary therapeutic reagents which may be used in combination with the polypeptides of this invention are the various interferons, especially gamma interferon, B cell growth factor, IL-1 and the like. For therapeutic or diagnostic applications, they may be formulated in nontoxic, nonallergenic, physiologically compatible carrier media such as distilled water, Ringer's solution, Hank's solution, physiological saline and the like. Administrations of the IL-2 muteins to humans or animals may be oral or intraperitoneal or intramuscular or subcutaneous as deemed appropriate by the physician. The amount of IL-2 mutein administered will usually range between about 1×10^4 and 2×10^8 units.

Modifications of the above described modes for carrying out the invention that are obvious to those of skill in the fields of genetic engineering, protein chemistry, medicine, and related fields are intended to be within the scope of the following claims.

CM We claim:

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